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Hormones and bile acids as biomarkers for the characterization of animal management in prehistoric sheepfold caves: El Mirador case (Sierra de Atapuerca, Burgos, Spain)

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ABSTRACT

Early husbandry practices that include herd management and the use of livestock areas such as sheepfold caves can be analysed in the context of different disciplines (e.g. zooarchaeology, micromorphology, and archaeobotany). In this study, a new and standard method for the determination of bile acids and steroidal hormones that incorporates microwave extraction-liquid chromatography-mass spectrometry was used. This method has been applied successfully to analyse Neolithic fumier deposit facies from the El Mirador cave, a location that was used as a prehistoric sheepfold and is located in the Atapuerca range (Burgos, Spain). The results obtained demonstrated that the analysis of bile acids can be useful for the identification of remains of ruminant residues in the facies studied. In addition, the progesterone/deoxycholic acid ratio has been used as a possible biomarker to improve our understanding of flock management, including the separation of pregnant and nursing ewes from the rest of the herd to avoid the rejection of the lamb and keep them safe and healthy.

1. Introduction

The domestication of animals expanded throughout the Mediterranean Basin from the 8th millennium cal BCE (Ethier et al., 2017; Gerbault et al., 2014; Larson et al., 2007; Zeder, 2008), finally reaching the Iberian Peninsula in the second half of the 6th millennium cal BCE (Oms et al., 2016; García-Puchol et al., 2017). In this context, caves and rock shelters began to be used as livestock pens. One common shepherding practice used throughout the Mediterranean from the Neolithic to the Bronze Age was the burning of the accumulated dung in the sheepfold caves and rock shelters to clean these locations, eliminate parasites, and reduce dung volume (Angelucci et al., 2009; Fernández-Eraso and Polo-Díaz, 2008; Polo-Díaz et al., 2014). This continuous burning produced an accumulation of various organic and mineral sediment layers that are commonly referred to as *fumiers*. Fumiers are formed by alternating layers of burned and unburned dung layers or *facies* along with different polychromes that were produced by the different thermal expositions of the dung during each burning (Polo-Díaz and Fernández-Eraso, 2010; Vergès et al., 2016a, 2016b).

Fumier deposits were identified according to micromorphological analyses of spherulites, phytoliths, and freshwater diatoms that are related to animal droppings in combination with analyses of archaeobotanical remains (e.g. charred wood and seeds, ashes, and phytoliths), linked to animal feeding and droppings (Angelucci et al., 2009; Brochier et al., 1992). These studies in combination with zooarchaeological analyses of the faunal remains indicated that the primary species bred in the sheepfold caves were sheep and goats (e.g., Brochier et al., 1992; Helmer et al., 2005). Other criteria for identifying the use of these areas as sheepfolds include the rock polish produced by sheep and goat rubbing and the presence of enclosing structures (Brochier et al., 1992; Vergès and Morales, 2016).

The favourable conditions of preservation of the thermically non altered facies allow the composition of the organic matter found in these caves to survive and ultimately provide a source of information

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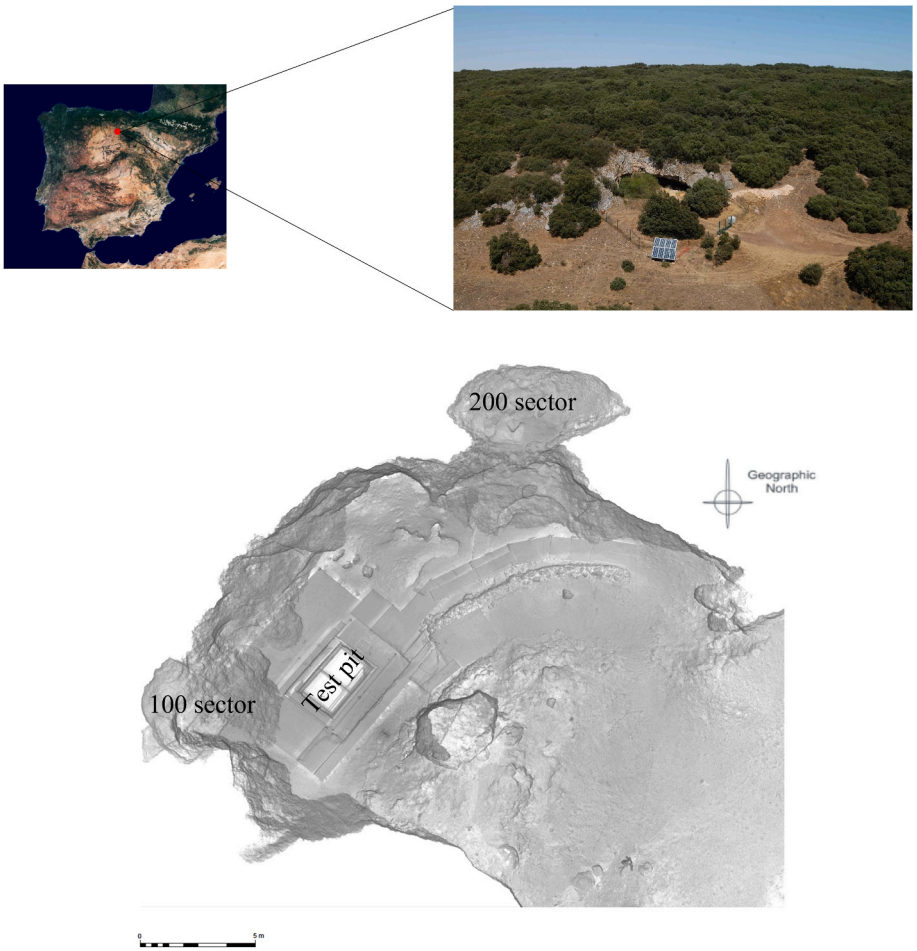

Fig. 1. El Mirador cave location and sectorial distribution.

regarding ancient husbandry practices and behaviours (Angelucci et al., 2009). One strategy used to obtain information from these *facies* deposits is the study of biomarkers, particularly faecal biomarkers (Evershed, 2008; Evershed et al., 1999).

Bile acids are faecal biomarkers derived from vertebrate animals, and their composition differs significantly among species. They are synthesised in the liver as primary bile acids, stored in the gallbladder, and secreted into the intestinal lumen. The functions of bile acids include emulsifying fats, increasing lipase activity, maintaining cholesterol levels, and aiding in lipid absorption. Additionally, once present in the gut, these acids are metabolised into secondary bile acids by the action of the intestinal microbiota, and the vast majority of these secondary bile acids is reabsorbed. Only a small percentage (~5%) is excreted (Matysik et al., 2016; Nasini et al., 2013). Nevertheless, the excreted amount is sufficient for faecal source assignment. Ruminants exhibit the highest percentage of deoxycholic acid (Deox), while omnivores possess more lithocholic acid (Lit). Hyodeoxycholic and Ursodeoxycholic acid (Urso) are specific to pigs (Bull et al., 2002; Prost et al., 2017). Based on this, it has been established that when Deox is present at 10-fold or greater amounts than is Lit (Deox/Lit >10), the sample originated from ruminants (Gea et al., 2017; Prost et al., 2017)

These faecal biomarkers are all related to the metabolism and the diet, and they are therefore useful for identifying the type of domestic livestock housed in the cavity in combination with contextual data provided by micromorphological analysis. Prost et al. (2017) proposed a bioarchaeological identification of animal species based on bile acids and 5β-stanols ratios and proxies. They were able to classify human, porcine, horse, donkey, sheep/cattle, goat, and goose faeces.

Unfortunately, these biomarkers cannot provide data regard to herd distribution because they are just dependant on the diet, which can be related to the animal species but not to herd distribution (Bull et al., 2003; Gea et al., 2017; Prost et al., 2017, 2018; Vázquez et al., 2021).

In addition to these analyses, the study of steroidal hormones (androgens, oestrogens, and progestins) can offer information regarding herd management. These faecal metabolites have been used to gather information regarding the oestrous cycle, pregnancy, abortion, puberty, and reproductive behaviour in farm, wild, and zoo animals (Kumar et al., 2013; Ruiz-Cortés, 2012; Schwarzenberger et al., 1996; Wang et al., 2016). This is possible based on the knowledge that the faecal hormone concentration truly correlates to those observed in the serum obtained after the centrifugation of the blood, during the gestational age (Capezzuto et al., 2008). The primary androgen hormone, testosterone (TT), exhibits seasonality and represents social dominance in males (Gesquiere et al., 2014; Shargal et al., 2008). Oestrogens (oestriol [E3], 17β-oestradiol [E2], and oestrone [E1]) are the primary female sex hormones, and high peaks in these hormones can be used to confirm pregnancy. Finally, progesterone (PG) is the major hormone in females and is involved in the menstrual cycle, pregnancy, and embryogenesis (Croxatto, 2017). In contrast to other sex hormones that are excreted through bile, faeces, and urine, PG can be partially metabolised into 5α and 5β pregnanes prior to excretion (Schwarzenberger et al., 1996); however, PG is detected at high concentrations in faeces and urine (Ahuja-Aguirre et al., 2017; Isobe et al., 2007; Shargal et al., 2008). To date, no standard methodology for the simultaneous analysis of bile acids and sex hormones from soil samples has been developed. Traditionally, bile acids have been extracted using organic solvents such as methanol/dichloromethane or chloroform/methanol via Soxhlet or sonication (Birk et al., 2012; Bull et al., 1999; Spangenberg et al., 2014).

Table 1
Samples collected from El Mirador cave. n.a.t. No altered thermally.

Sample	Sector	Level	Data (cal BC. 95.4%)	*Facies*
AM3	100	MIR104	1730–1530	charcoal (c)
AM4				tutti-frutti (tf)
AM21		MIR105	??	**a**
AM22				**a**
AM1	200	MIR205	3893–3661	grey (g)
AM2				**v (n.a.t)**
AM23				**vl (n.a.t)**
AM24				g
AM25				tf
AM26				tf
AM27				**v**
AM5	100	MIR107	4542–4371	white (w)
AM6				tf
AM7				c
AM8				g
AM9				brown (b)
AM10				c
AM11				b
AM12				g
AM13				w
AM14				tf
AM15				c
AM16				g
AM17				tf
AM18				c
AM19		MIR108	4498–4361	**v**
AM20				**vl**
AM28				**v**
AM29				**vl**
AM30				g
AM31				**a**
AM32				**a**
AM33		MIR109	4836–4700	w

Table 2
High and low concentration recoveries (%), repeatability (relative standard deviation, RSD [%]), and limits of detection (C_{LOD}, ng/g).

Analytes	Low conc. recoveries (%) 2 x C_{LOD}	RSD (%)	High conc. recoveries (%) 10 x C_{LOD}	RSD (%)	C_{LOD} (ng/g)
E_1	71	8	123	13	8
E_2	82	15	112	2	2
E_3	95	10	107	3	9
TT	92	21	100	19	4
PG	102	19	74	24	10
Urso	127	9	78	12	11
Hyo	105	13	86	10	3
Cheno	111	24	120	10	0.5
Deox	78	10	65	9	13
Lit	119	12	129	9	9

Subsequently, the total lipid fraction is concentrated and saponified, and the saponified extract is then separated using a liquid-liquid extraction into neutral and acidic fractions. The acidic fraction includes bile acids, and a further clean-up step is commonly performed using solid-phase extraction cartridges. Finally, both fractions are derivatized and analysed using gas chromatography-mass spectrometry (GC-MS) (Birk et al., 2012; Bull et al., 2002; Nasini et al., 2013; Sistiaga et al., 2014). In contrast, sex hormones are extracted using solvents with greater polarity or by centrifugation, and their concentrations are typically determined using immunoassays (Capezzuto et al., 2008; Isobe et al., 2007; Kumar et al., 2013).

These aspects are all particularly interesting for the study of herd management in caves and rock shelters. It has been observed that many of these locations contain a high number of foetal and neonatal sheep and goat remains (Boscchini and Riedel, 2000; Martín et al., 2016; Miracle, 2006; Mlekuž, 2005; Radovic et al., 2008). In the case of El Mirador cave (Sierra de Atapuerca, Spain), certain authors have suggested that this abundance could be related to the possible use of these sites as breeding and birthing areas (Martín-Rodríguez and Vergès, 2016). The direct study based on the presence/absence and the quantification of hormone levels in soils will allow us to confirm or discard the hypotheses proposed based on zooarchaeological criteria. Therefore, the aims of this study were (i) to develop a new methodology based on microwave extraction (MAE) and liquid chromatography-mass spectrometry (LC-MS) analysis that can be used for the rapid and simultaneous detection and quantification of four bile acids and five hormones, (ii) to quantify and study the faecal biomarkers from the Neolithic samples found in the El Mirador cave (Sierra de Atapuerca, Spain) to determine the origin of that faecal input, and (iii) to verify if the quantity of sex hormones can provide information according to herd distribution.

2. Material and methods

2.1. The site

The El Mirador cave is located on the south side of Sierra de Atapuerca, Burgos, Spain (see Fig. 1. 42°20′58″ N and 03°30′33″ W).

A test pit of 6 m^2 was excavated at the entrance area of El Mirador Cave from 1999 to 2009 (see Fig. 1). The results together with detailed chronological characterization of the stratigraphy allowed the identification of the site as sheepfold during the Neolithic period (second half of 6th millennium cal BCE to the first half of the 4th millennium cal BCE) and the Bronze Age (2nd millennium cal BC) (Vergès et al., 2016a). Currently, the excavation work is focused on other two sectors (100 and 200) that are located on the west and north sides of the cave, respectively. To date, eight archaeological levels have been identified in sector 100, and all of these were identified as Neolithic and Bronze Age *fumier* deposits. In sector 200, seven archaeological levels were identified. MIR203 possesses a Chalcolithic chronology and corresponds to the funerary use of the cave. In contrast, from the MIR204 level onwards a Neolithic occupation develops with *fumier* deposits.

Sedimentary *facies* were used to document the complexity of the *fumier* deposits. These *lithofacies* were identified and named according to their features (e.g. colour, composition, and thermal alteration) (Vergès et al., 2016a).

Archaeobotanical and zooarchaeological studies have revealed the development of a mixed economy, agriculture, and livestock (Expósito and Burjachs, 2016; Expósito et al., 2017; Martín et al., 2016; Rodríguez et al., 2016). Conversely, zooarchaeological studies have focused on the remains recovered in the initial excavated area (MIR24-MIR3) and at several levels from sector 100 (MIR105-MIR103). According to these studies, husbandry was based on the breeding of sheep and goats. Additionally, large numbers of foetal and neonatal individuals were found, particularly at the Neolithic levels. Based on these data and the amplitude and orientation of the cave among the other caves, the use of El Mirador was proposed to be used for the housing of pregnant females, likely to separate them from the rest of the herd (Martín et al., 2016). The abundance of perinatal remains is not exclusive to the El Mirador cave. Other caves holding *fumier* deposits have also presented high percentages of perinatal remains such as Grotta dell'Edera (north-eastern Italy) (Boschin, 2019) and Cova de L'Or (eastern of the Iberian Peninsula (Pérez-Ripoll, 2016)).

2.2. Sampling, storage, and homogenisation of the samples

For this study, thirty-three samples were collected from *fumier* deposits and relevant facies type of both sectors (26 from sector 100 and 7 from sector 200). These *facies* were classified according to radiocarbon data and thermal alteration (see Table 1). Thermal alteration colours have been described in detail by Vergès et al. (2016a)

The *facies* were collected from rectangular blocks that were 0.8 m in length and 0.6 m in width using a stainless-steel trowel that was cleaned with MeOH and Milli-Q water after each use. Each sample was stored in

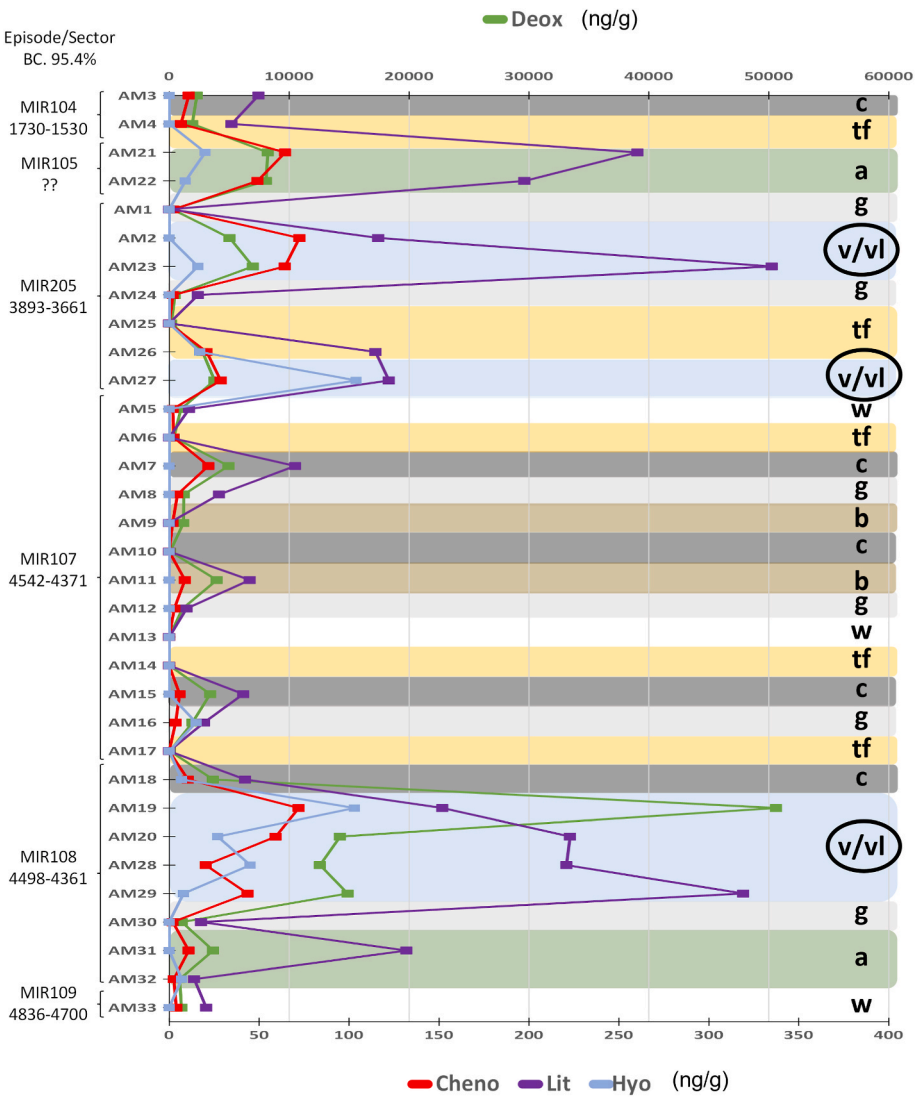

Fig. 2. Concentrations of detected target compounds classified according to our data. Deoxicholic acid is presented on the y-axis. Thermally unaltered *facies* are circled.

aluminium foil, transported to the laboratory in a zip-bag at 4 °C, and maintained at −82 °C until analysis. Prior to analysis, all samples were crushed using a mortar and pestle, dried at 90 °C in an oven for 12 h, and sieved to a particle size fraction of 60 μm.

2.3. Chemical, reagents, and reference standards

Hormones, (17β-oestradiol [E_2, 99.7%], oestrone [E_1, 99%], testosterone [TT, 99.9%], and progesterone [PG, 99.6%]), bile acids (ursodeoxycholic acid [Urso, 99%], hyodeoxycholic acid [Hyo, 99%], chenodeoxycholic acid [Cheno, 97%], deoxycholic acid [Deox, 98%], and lithocholic acid [Lit, 95%]), isotopically labelled E_2 ([$^2H_3$]-E2, 98%), and Lit ([$^2H_5$]-Lit, 98%) were supplied by Sigma-Aldrich (Madrid, Spain), and oestriol hormone (E_3, 99.7%) was obtained from Riedel-de Haën (Streiheim, Germany).

Solvents such as methanol (MeOH, HPLC grade), dichloromethane (DCM, HPLC grade), ethyl acetate (EtOAc HPLC grade), and potassium hydroxide (KOH) were purchased from Scharlab (Barcelona, Spain). Finally, hydrochloric acid (HCl) was purchased from Fisher Scientific (Loughborough, UK).

Ultra-high purity water was obtained from tap water that was pretreated by Elix reverse osmosis and subsequent filtration using a Milli-Q system from Millipore (Bedford, MA, USA) and was used in the saponification step and for cleaning.

2.4. Microwave assisted extraction (MAE)

Homogenised sample extraction was performed according to a previous study that was optimised by Gea. et al. (Gea et al., 2017). Briefly, 5 g of homogenised sample was weighed into each microwave vessel, and after the addition of 150 ng of the deuterated analogues, 25 mL of a DCM:MeOH (2:1 v/v) mixture was added. Next, the extraction was performed in a microwave oven at 1600 W (MAE, CEM Mars 5, Mathews, NC, USA) by increasing the temperature to 150 °C over a 10 min time period and then maintaining this temperature for 10 min while stirring. Once the extraction step was complete, the extract was centrifuged for 5 min at 10,000 rpm, and the supernatant was then removed. The extract was evaporated to dryness under a gentle stream of nitrogen prior to saponification.

2.5. Saponification and liquid-liquid extraction

The dried extracts were saponified by adding 5 mL of 2 M KOH (MeOH:H_2O, 10:1 v/v) and the incubating was performance at 100 °C for 90 min. Next, 5 mL of 2 M HCl/H_2O was added to the saponified extracts to acidify the solution ($pH = 2 \leq x \leq 4$). Then, continuous

Table 3
Classification of the *facies* according to the equation. n.a.t., not altered thermally.

Level	Sample	Deox/Lit	*Facies* colour
MIR104	AM3	Ruminant (46)	charcoal (c)
	AM4	Ruminant (55)	tutti-frutti (tf)
MIR105	AM21	Ruminant (32)	**a**
	AM22	Ruminant (41)	**a**
MIR205	AM1	Ruminant (No LIT)	grey (g)
	AM2	Ruminant (43)	**v (n.a.t)**
	AM23	Ruminant (21)	**vl (n.a.t)**
	AM24	Ruminant (30)	g
	AM25	Ruminant (No LIT)	tf
	AM26	Ruminant (24)	tf
	AM27	Ruminant (31)	**v**
MIR107	AM5	Ruminant (89	white (w)
	AM6	Ruminant (No LIT)	tf
	AM7	Ruminant (71)	c
	AM8	Ruminant (44)	g
	AM9	Ruminant (No LIT)	brown (b)
	AM10	Ruminant (No LIT)	c
	AM11	Ruminant (89)	b
	AM12	Ruminant (115)	g
	AM13	??	w
	AM14	??	tf
	AM15	Ruminant (83)	c
	AM16	Ruminant (100)	g
	AM17	Ruminant (No LIT)	tf
	AM18	Ruminant (87)	c
MIR108	AM19	Ruminant (333)	**v**
	AM20	Ruminant (64)	**vl**
	AM28	Ruminant (57)	**v**
	AM29	Ruminant (47)	**vl**
	AM30	Ruminant (59)	g
	AM31	Ruminant (28)	**a**
	AM32	Ruminant (63)	**a**
MIR109	AM33	Ruminant (50)	w

liquid-liquid extraction with DCM (2 × 10 mL) was performed. The lower organic phase was collected and evaporated to dryness under a gentle stream of nitrogen. The extract was dissolved in 250 μL of MeOH. Finally, after centrifugation for 5 min at 13,000 rpm (Centrifuge 5415R, Eppendorf, Hamburg, Germany), the supernatant was transferred to chromatography vial inserts.

2.6. Instrumental analysis

Samples were analysed on an Agilent 1290 infinity liquid chromatography system (Palo Alto, SA, USA) consisting of a binary pump, autosampler, and Infinity TCC oven modules. Chromatographic separation was performed using an Ascentis Express C_{18} HPLC column (15 cm, 3.0 mm, 2.7 μm; Supelco, Sigma-Aldrich). The temperature of the column was set to 35 °C. The mobile phase consisted of a gradient elution phase. Solvent A was Milli-Q water, and solvent B was MeOH. The elution of the analytes was achieved using the following solvent gradient: 0 min 35% A and 65% B; 3 min 26.3% A and 73.7% B; 4 min 25.0% A and 75.0% B; 8 min 15.0% A and 85.0% B; 10 min 1.6% A and 98.4% B; 20 min 0.9% A and 99.1% B; 22 min 0.0% A and 100% B; 23 min 35.0% A and 65.0% B. The flow rate of the mobile phase was set to 0.6 mL/min, and the injection volume was 1 μL.

A mass spectrometer (Agilent 6120) was used for atmospheric pressure chemical ionisation (APCI). The scan event was conducted in the positive-ion mode. The source conditions were as follows: capillary voltage, 3000 V; drying gas temperature, 300°C; drying gas flow, 3.0 L/min; nebuliser gas pressure, 20 psig. Measurements were performed in the selected ion monitoring (SIM) mode. The first ion was used as the quantifier and the second as the qualifier for all compounds (E3: 271.1/253.1; [$^2H_3$]-E2: 258.2/249.1; E2: 255.2/271.0; E1: 271.1/253.1; TT: 289.2/271.1; Urso: 357.2/358.2; PG: 315.2/313.1; Hyo: 357.2/358.2; Cheno: 357.2/339.1; Deox: 357.2/355.0; [$^2H_5$]-Lit: 364.3/346.3; Lit: 359.3/341.2).

3. Results and discussion

3.1. Quality parameters of the method

The method used for the determination of hormones and bile acids was based on a previously optimised method that was developed by Gea et al. (Gea et al., 2017). However, this method analysed Lit, Deox,

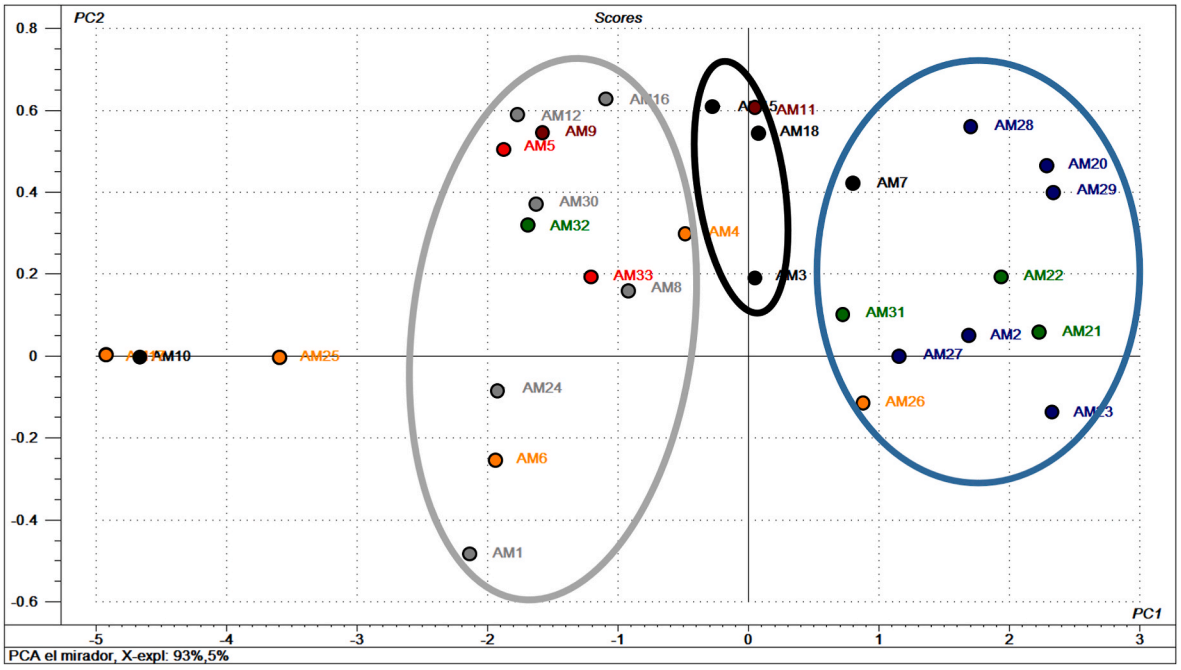

Fig. 3. Principal Component Analysis of the El Mirador cave *facies*. The bile acid concentrations (Deox, Lit, and Cheno) were normalised according to their logarithmic values. *Facies* colour description: Blue: v/vl, Green: a, Orange: tf, Red: w, Brown: b, Black: c, and Grey: g. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

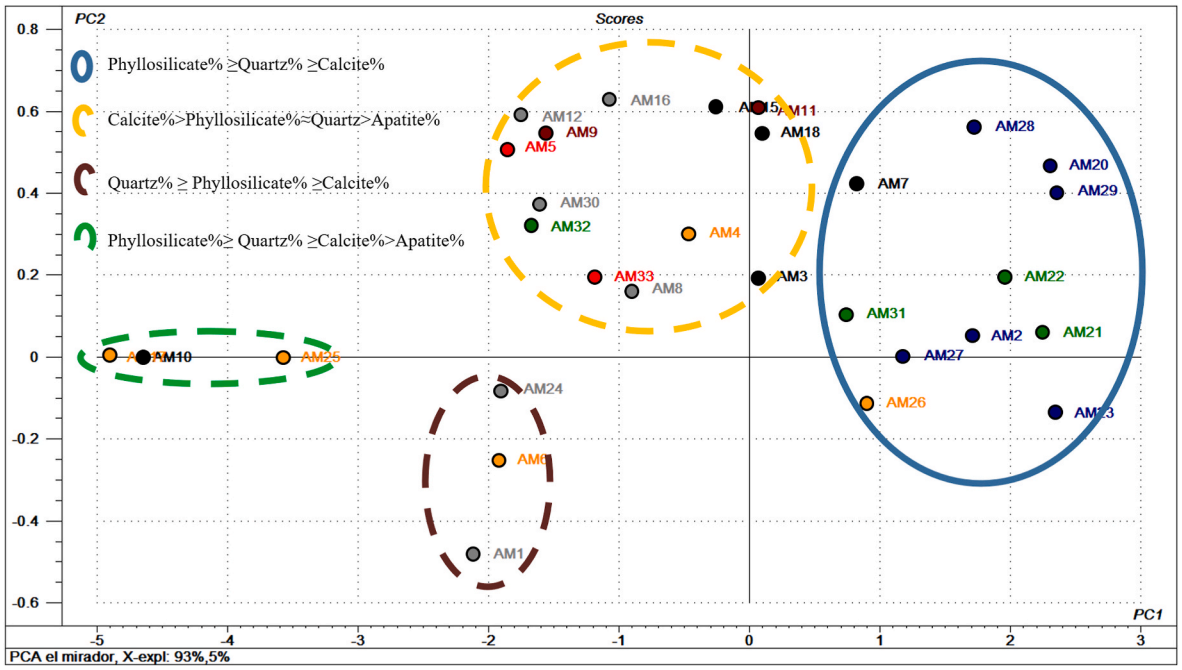

Fig. 4. Illustration of the mineral compositions of the different groups. *Facies* colour description: Blue: v/vl, Green: a, Orange: tf, Red: w, Brown: b, Black: c, and Grey: g. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sterols, and phytosterols using GC-MS. In our current study, the usefulness of the extraction method used by Gea et al. in combination with LC-MS analysis was assessed for E1, E2, E3, PG, TT, Urso, Hyo, Cheno, Deox and Lit.

Calibration of the analytes was performed using matrix blanks spiked with a 10–500 ng/g concentration range of each analyte and 50 ng/g of the deuterated analogues. Simultaneously, non-spiked matrix blanks were analysed to ensure the absence of the target analytes in the matrix. All correlation coefficients were greater than 0.99. The matrix effect was also studied, and deuterated analogues were used (see supplementary data). We observed matrix effects of 28% and 64% for [$^2H_5$]-LCA and [$^2H_3$]-E2, respectively. To minimise these effects, matrix match calibration was used.

High and low concentration recoveries, limits of detection (C_{LOD}), and the repeatability for each analyte were also calculated (see Table 2).

Acceptable recoveries were obtained for all analytes. An RSD% < 25% was observed for all cases, and C_{LOD} were always determined to exist at low ng/g levels.

3.2. Analysis of archaeological samples

3.2.1. Bile acid results and biogenic classification

As illustrated in Fig. 2 (for nominal values see Supplementary Data), the highest concentrations of bile acids were obtained from unburned and mixture of unburned/burned *facies* (a: AM21, AM22, and AM31; v/vl: AM2, AM19, AM20, AM23, AM27, AM28, AM29), and the lowest values (or no values) were obtained from burned samples (tf: AM4, AM25, AM14, and AM17; w: AM5, AM13, and AM33; g: AM8, AM12, and AM16). Partially burned *facies*, two of which were charcoal (AM7 and AM15) and one of which was brown (AM11), exhibited relatively high concentrations of bile acids, as did AM26 (tf). Additionally, MIR108 level samples (AM 19, AM20, AM28, AM29) presents the highest concentrations of bile acids, and this could be attributed to higher pastoral herding activity.

The abundance of Deox compared to the amount of Lit is related to the presence of ruminant faeces (Bull et al., 2002; Gea et al., 2017; Prost et al., 2017). As presented in Table 3, all of the ratio values between Deox and Lit (Deox/Lit) were higher than 20 with the exception of those values for AM13 and AM14. In the latter samples, bile acid values were below their C_{LOD} and could not be classified. Urso was not detected in any of the samples and Hyo at lower concentration than Deox and Lit in the unburned and mixture of unburned/burned facies at MIR105 (AM21 and AM22), MIR108 (AM19, AM20, AM28 and AM29) and, MIR205 (AM23, AM26, AM27). Based on this, the presence of pigs at low concentration in the cave is corroborate in these levels as Martín proposed (Martín, 2015). Hyo is specific biomarker of the pig, however, its concentration should be higher than Deox and Lit to ensure just pig dung and this does not happens (Prost et al., 2017). Therefore, the cave was used as shelter for ruminants, although pigs were also present in some cases. Other ratios were also calculated to determine the type of animal enclosed in the cave, and all of these ratios are still in disagreement with our results. Based on Prost et al. (2017), the presence of Cheno is related to goat, horse or goose. Besides, in the case of goat Lit and Cheno concentration must be similar and in the case of goose and horse, Cheno concentration higher than Lit. Nevertheless, in this study, any of those circumstances occur so we decided just to classify the faeces as ruminants.

The results obtained with Deox/Lit ratio are in agreement with the zooarchaeological and vegetal microremains analyses carried out in El Mirador cave, which confirm the abundance of ruminants and, in particular, ovicaprines in the herds (Cabanes et al., 2009; Martín et al., 2016; Burguet-Coca, 2020).

To illustrate the possible grouping of the samples, a principal component analysis (PCA) was performed based on the logarithmic concentration of bile acids (Deox, Lit, and Cheno). As presented in Fig. 3, 98% of all the samples were grouped into three categories that included unburned *facies* (blue), partially burned *facies* (black), and totally burned *facies* (grey). Loadings, influence, and explained variance are included in the supplementary material.

Nevertheless, certain *facies* such as AM26 (tf) o AM7 (c) were grouped with the unburned and the mixture of unburned/burned *facies*. This may be related to the chemical composition of the *facies*. Therefore, a semi-quantitative analysis of the selected *facies* was performed using X-ray diffraction analysis (XRD, see Supplementary Material). As expected, all of the *facies* were composed of calcite, phyllosilicate, quartz, and apatite at various percentages. However, the percentages were

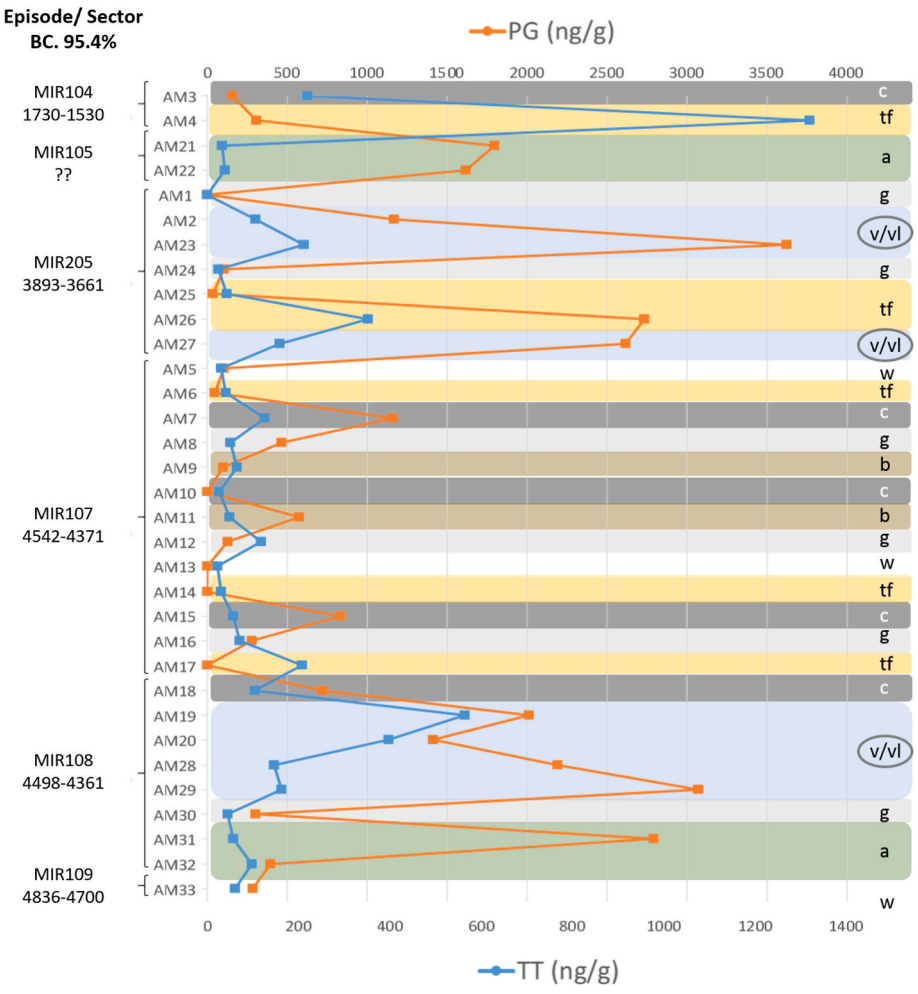

Fig. 5. Progesterone (PG) and Testosterone (TT) concentration profiles of the analysed *facies* from the archaeological site located in the Mirador cave. Thermally unaltered *facies* are circled.

determined to be related to the grouping that was observed in the PCA. As shown in Fig. 4, the grouped samples were related based on their percentage and abundance of calcite, phyllosilicate, quartz, and apatite. For example, the samples in the blue group were abundant in phyllosilicate, and this was followed by quartz and calcite. Additionally, the black and grey groups can be divided into two additional groups (yellow and brown) based on their mineral composition.

Based on the results obtained, the accumulation of the bile acids is related to the unburned facies and the mixture of unburned/burned facies, and consequently, to the mineral composition of that kind of facies which have high amount of phyllosilicate and quartz, less of calcite and very low or absence of apatite. Exceptionally, two facies with high thermal alteration, AM7 (c) and AM26 (tf) have also high amount of bile acids and similar mineral composition to unburned facies and mixture of unburned/burned facies. This behaviour can be related to a lower thermal alteration as expected, however, this hypothesis should be clarify with other kind of analysis such as IR, thermoremanece and micromorphology (Carrancho et al., 2016; Polo-Díaz et al., 2016; Polo-Díaz and Fernández-Eraso, 2010) Thus, XRD analysis should be performed prior to bile acid analysis to determine which samples could accumulate bile acids, as this can be performed independently of the visual criteria.

3.2.2. *Hormone analysis and identification of the activity of shepherds*

PG and TT are the only hormones that were detected in the *facies* analysis. These compounds are the most hydrophobic compounds derived from the studied hormones, and they are more persistent in manure according to previous studies (Zhang et al., 2015, 2019). E1, E2, and E3 concentrations were always present at levels that were below their limits of detection. The absence of the latter three hormones may be due to different factors. Zhang et al. did not observe E3 in cattle and sheep manure, however, we know herbivore dung from at least sheep-goat were sources of a number of our samples as indicated by micromorphological analysis. Alternatively, preservation issues related to taphonomy could explain the absence of these analytes in the sediment of El Mirador: first, E3 is highly soluble in water, and it has therefore been determined to be easily leached (Zhang et al., 2014, 2015). In addition, E2 is easily oxidised to E1 in manure, and its persistence in dung is therefore very low. Based on this, its presence in *fumier* deposits is questionable (Bartelt-Hunt et al., 2013; Hakk and Sikora, 2011; Ma and Yates, 2017). Finally, the dissipation of E1 by leaching is difficult according to its distribution coefficient ($K_d = C_{soil}/C_{water}$), and E1 also exhibits lower degradation rates than do E2 and E3 (Xuan et al., 2008; Zhang et al., 2015). However, some studies described a high mineralisation of E1 by microorganisms at an appropriate temperature and moisture, and this could be the underlying reason for the absence of this compound in the samples (Colucci et al., 2001).

Fig. 5 illustrates the PG and TT concentration profiles observed for the different *facies* and their different levels.

As presented in Fig. 5, the highest amount of PG was detected in the unburned *facies* v/vl (AM2, AM23, AM27, AM19, AM20, AM28, and

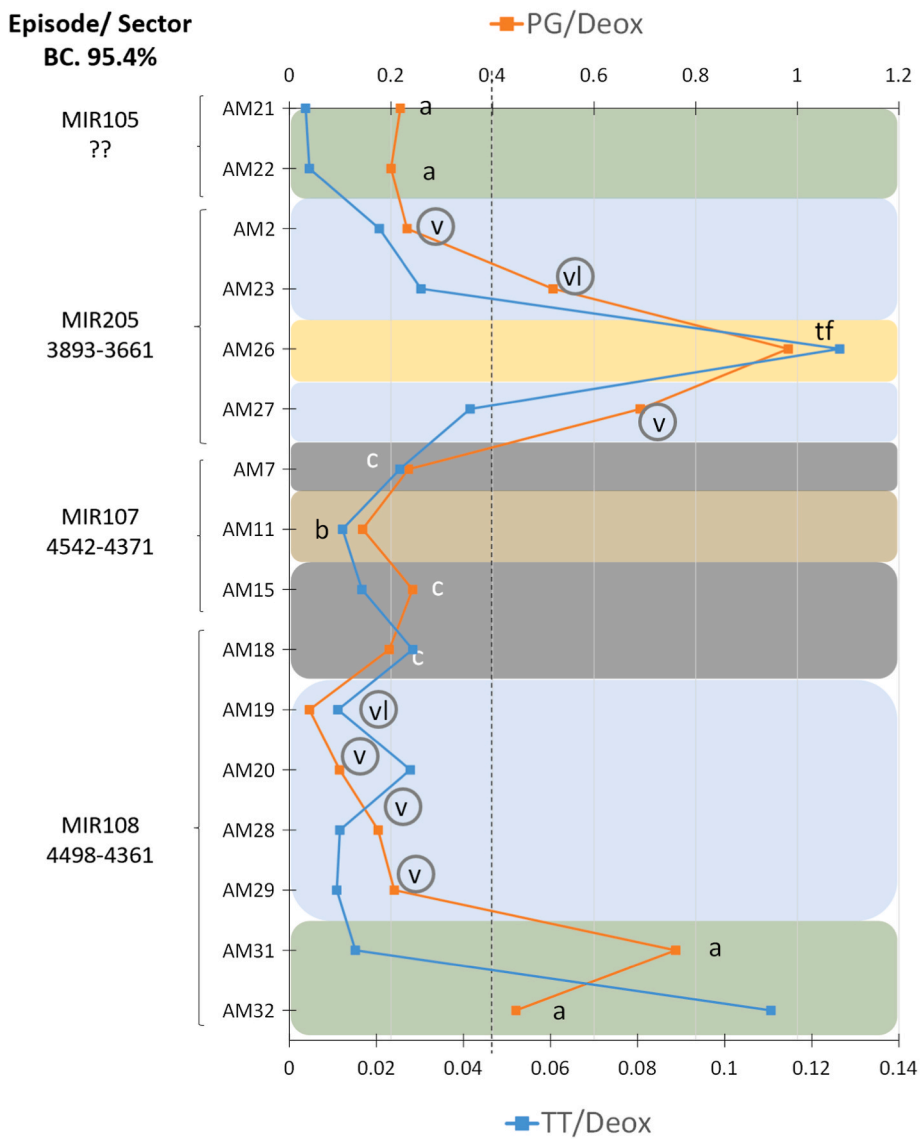

Fig. 6. PG/Deox and TT/Deox ratios for v/vl, a, c and b *facies* obtained from the El Mirador archaeological site. Thermally unaltered *facies* are circled.

AM29), mixture of unburned/burned facies a (AM21, AM22, and AM31) and tf (AM26). The latter *facie* (tf) correspond to the tf *facie* that were grouped with the unburned *facies* (see Fig. 4). Lower concentrations of PG were observed in partially burned *facies* c (AM7, AM15, and AM18) and b (AM11), and the lowest concentrations were detected in completely burned *facies* g (AM1, AM24, AM8, AM12, AM16, and AM30), w (AM5, AM13, and AM33), and tf (AM4, AM25, AM6, AM14, and AM17).

The results for TT were similar to those for PG, where the highest concentrations were detected in unburned *facies* v/vl (AM23, AM27, AM19, AM20, AM28, and AM29) and in two facies tf (AM26 and AM4). Interestingly, the AM4 sample possessed yielded the highest amount of TT of all the *facies*. Medium and low concentrations were observed in the remaining partially and totally burned *facies* c (AM3, AM7, AM15, and AM18), tf (AM25, AM6, AM14, and AM17), g (AM1, AM12, and AM30), and w (AM5, AM13, and AM33).

As presented in Fig. 5, a similar profile was observed for all v/vl and b *facies* in regard to both hormones. These concentration variations could be related to individual shepherding practices. For example, an increase or decrease of livestock in the pen could provide a reason for the growth or decline of hormone concentrations in the *facies*. Additionally, these variations could be related to livestock management involving the separation of pregnant females or recent lamb ewes and their lambs from the herd that could result in an increase in the hormonal concentration in the sediment of specific levels. In regard to the zooarchaeological study of MIR108 and MIR205 levels analysed in the present study, these data were consistent with the abundance of perinatal remains documented in MIR24-MIR18 and MIR8-MIR9 levels (Martín et al., 2016) that hold a similar chronology to that of MIR108 and MIR205, respectively.

Ethnographic studies also documented these practices that allow for better care of pregnant females by the shepherd as well as better assistance with deliveries and care of the lambs, thus avoiding lamb rejection (Cambero, 1997; Halstead, 1998; Repesa, 1998).

To eliminate the effect of livestock head, PG and TT concentrations were normalised to the concentration of Deox that was related to the amount of residue produced by the herd. Fig. 6 illustrates the normalised hormone profiles for the non-burned and partially burned *facies*. Grey and white *facies* were not illustrated due to the low concentration of bile acids produced at high temperatures.

The PG/Deox ratio was increased in the AM23, AM26, and AM27 *facies* from sector 200 and in the AM31 and AM32 *facies* from sector 100. This increase could also be related to the separation of pregnant females or recent lamb ewes and their lambs from the herd. Both activities

resulted in higher excretion concentration of PG excreted through the faeces and urine.

The TT/Deox ratio exhibited an increase in hormonal activity in AM27 and AM32. Although TT is commonly related to male activity, it is also present in females at much lower concentrations. Due to the lower concentration of TT in the samples compared to the concentration of PG, it was more difficult to observe clear variations such as those observed with PG. Therefore, the PG/Deox ratio was selected as the most optimal parameter by which to observe these variations.

These results demonstrate a higher hormonal activity in AM23, AM26, AM27, AM31, and AM32 *facies* that could be related to the separation of pregnant females or recent lamb ewes and their lambs from the herd or to the use of El Mirador cave as a shelter for pregnant females. As described above, the abundance of perinatal remains is not exclusive to the El Mirador cave, and this methodology could therefore be applied to other cave and rock-shelter herding sites to investigate their use for similar purposed.

4. Conclusions

This work demonstrated that the analysis of organic residues is a study approach that can be useful in archaeology for characterising animal species and for clarifying animal management and domestication during early farming. The analysis of biomarkers such as bile acids allows for identification of the animals that were housed in a given sheltered in caves and rock-shelters, however, uniquely in this study, sexual hormones such as PG or TT were also studied as possible new biomarkers to allow for further understanding of prehistoric herding practices between the Neolithic and the Bronze Age.

A new and standard methodology was developed for the analysis of five sex hormones (E1, E2, E3, TT, and PG) and five bile acids (Lit, Deox, Urso, Hyo, Cheno) using the microwave extraction technique and followed by LC-MS analysis. The samples analysed were characterised as animal faeces, and most were derived from ruminants. Our findings confirmed that the El Mirador cave was used as sheepfold and are in agreement with the results of previous studies that used zooarchaeological analysis and micromorphology of soils. Unburned *facies* were useful for determining the animal origin of the samples, while the use of the other samples could provide misleading results; however, an initial *facies* analysis using XRD should be performed to determine which samples should be further analysed to improve on the visual criteria that are currently used.

The use of PG and TT as biomarker for herding practices was explored in this study. Human management of the livestock involving separation of the pregnant females or the recent lamb ewes and their lambs from the herd has been inferred as a plausible explanation for the observed the increase in the PG/Deox ratio documented in the sedimentary *facies*. This novel biomarker ratio provides new data regarding the herding practices that is not possible to obtain by micromorphological studies, and our findings are complementary to those provided by zooarchaeological studies which corroborates that the pregnant females and their offspring were stabled in the cave.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2022.105547.

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